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(54) Title: PATHOGEN-INDUCIBLE PROMOTER <p style="text-align: center;">ms59 regulatory region</p> <p>— = part sequenced</p> <p>- - - = ms59 coding region</p>			
(57) Abstract This invention describes pathogen-inducible promoters which normally drive expression of plant hexose oxidases, especially those which can be isolated from <i>Helianthus annuus</i> and <i>Lactuca sativa</i> , more specifically those promoters which naturally are the regulatory regions driving expression of the hexose oxidase MS59 and WL64, respectively. Also claimed are chimeric constructs where these pathogen-inducible promoters drive expression of antipathogenic proteins or of proteins which can elicit a hypersensitive response.			
Bao, et al. App. No. 09/923,844			REF A20

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PATHOGEN-INDUCIBLE PROMOTER

FIELD OF THE INVENTION

5 This invention is related to the field of pathogen-inducible promoters, and chimeric DNA sequences comprising said promoters, especially in the area of plant biotechnology.

BACKGROUND ART

10 Inducible promoters include any promoter capable of increasing the amount of gene product produced by a given gene, in response to exposure to an inducer. In the absence of an inducer the DNA sequence will not be transcribed. Typically, the factor that binds specifically
15 to an inducible promoter to activate transcription is present in an inactive form which is then directly or indirectly converted to the active form by the inducer. The inducer may be a chemical agent such as a protein, metabolite (sugar, alcohol, etc.), a growth regulator, herbicide, or a phenolic compound or a physiological stress imposed
20 directly by heat, salt, wounding, toxic elements etc., or indirectly through the action of a pathogen or disease agent such as a virus. A plant cell containing an inducible promoter may be exposed to an inducer by externally applying the inducer to the cell such as by spraying, watering, heating, or similar methods. Inducible promoters
25 are known to those familiar with the art and several exist that are be used to drive expression of genes of interest. Examples of inducible promoters include the inducible 70 kD heat shock promoter of *Drosophila melanogaster* (Freeling, M. et al., Ann. Rev. Genet. 19, 297-323) and the alcohol dehydrogenase promoter which is induced by
30 ethanol (Nagao, R.T. et al., in: Miflin, B.J. (ed.) Oxford Surveys of Plant Molecular and Cell Biology, Vol. 3., pp. 384-438, Oxford Univ. Press, 1986). Examples for promoters that are inducible by a simple chemical are the promoters described in WO 90/08826, WO 93/21334, WO 93/031294 and WO 96/37609.

35 An important subclass of inducible promoters are the promoters which are induced in plants upon pathogen infection. As examples of a pathogen-inducible promoter the PRP1 promoter (also named *gst1* promoter) obtainable from potato (Martini N. et al. (1993), Mol. Gen.

Genet. 263, 179-186), the *Fis1* promoter (WO 96/34949), the *Bet v 1* promoter (Swoboda, I., et al., Plant, Cell and Env. 18, 865-874, 1995), the *Vst1* promoter (Fischer, R., Dissertation, Univ. of Hohenheim, 1994; Schubert, R., et al. Plant Mol. Biol. 34, 417-426, 1997), the sesquiterpene cyclase promoter (Yin, S., et al., Plant Physiol. 115, 437-451, 1997) and the *gstA1* promoter (Mauch, F. and Dudler, R., Plant Physiol. 102, 1193-1201, 1993) may be mentioned. A drawback of some of these promoters is that they are also active constitutively or that they do not react to certain types of pathogens. Furthermore, it would be advantageous to have promoters that regulate expression very soon after pathogen infection, i.e. with as short as possible induction times.

Thus, there is still need for promoters that are pathogen-inducible which overcome the disadvantages of the prior art.

SUMMARY OF THE INVENTION

We now have found DNA fragments which are the upstream regulatory regions for plant genes coding for hexose oxidase, capable of promoting pathogen-inducible transcription of an associated DNA sequence when re-introduced into a plant. Preferably such a fragment is obtainable from *Helianthus annuus*. Said DNA fragment specifically is the upstream regulatory region of the gene coding for hexose oxidase, denoted as MS59, more specifically characterized in that it comprises the nucleotide sequence from 1 to 1889 depicted in SEQ ID NO: 15.

Also part of the invention is a DNA fragment obtainable from *Lactuca sativa*, capable of promoting pathogen-inducible transcription of an associated DNA sequence when re-introduced into a plant, specifically the DNA fragment that it is the upstream regulatory region of the gene coding for hexose oxidase, denoted as WL64 (SEQ ID NO: 18).

Also included in the invention is a portion or variant of a DNA fragment according to any described above, capable of promoting pathogen-inducible transcription of an associated DNA sequence when re-introduced into a plant.

Embodiments of the invention are chimeric DNA sequences comprising in the direction of transcription a DNA fragment according to any one of the DNA fragments described above and a DNA sequence to be expressed under the transcriptional control thereof and which is not naturally under transcriptional control of said DNA fragment.

A preferred embodiment is such a chimeric DNA sequence wherein the DNA sequence to be expressed causes the production of an antipathogenic protein, which is preferably selected from the group consisting of chitinase, glucanase, osmotin, magainins, lectins, saccharide oxidase, oxalate oxidase, toxins from *Bacillus thuringiensis*, antifungal proteins isolated from *Mirabilis jalapa*, *Amaranthus*, *Raphanus*, *Brassica*, *Sinapis*, *Arabidopsis*, *Dahlia*, *Cnicus*, *Lathyrus*, *Clitoria*, *Allium* seeds, *Aralia* and *Impatiens* and albumin-type proteins, such as thionine, napin, barley trypsin inhibitor, cereal gliadin and wheat-alpha-amylase.

Another embodiment of the chimeric DNA sequences of the invention is a chimeric DNA sequence wherein the DNA sequence to be expressed causes the production of a protein that can induce a hypersensitive response, preferably selected from the group consisting of CF and Pto proteins from tomato, avr proteins from *Cladosporium fulvum* and elicitor proteins from *Pseudomonas* or *Xanthomonas*.

Further part of the invention are replicons comprising above mentioned chimeric DNA sequences preferably having at least one recognition site for a restriction endonuclease for insertion of a DNA sequence to be expressed under the control of said DNA fragment. Also included in the invention are microorganisms containing such a replicon, plant cells having incorporated into their genome a chimeric DNA sequence according to those described above, and plants essentially consisting of said cells. Such a plant is preferably a dicotyledonous plant. Also part of said plants selected from seeds, flowers, tubers, roots, leaves, fruits, pollen and wood, form part of the invention.

Yet another embodiment of the invention is the use of a DNA fragment as described above for identifying homologues capable of promoting pathogen-induced transcription in a plant.

Further use of a chimeric DNA sequence according to the invention for transforming plants and use of a portion or variant of

the DNA fragments according to the invention for making hybrid regulatory DNA sequences is part of the invention.

Another object of the invention is the use of a chimeric DNA sequence as described above for conferring pathogen resistance to a plant.

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DESCRIPTION OF THE FIGURES

Fig. 1. Schematic drawing of (genomic) ms59 gene and promoter region.

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DETAILED DESCRIPTION OF THE INVENTION

The main aspect of the invention are regulatory sequences naturally occurring in plants and driving the expression of genes coding for hexose oxidase. Specifically the regions occurring in
10 *Helianthus annuus* and/or *Lactuca sativa*, especially in the 5' (upstream) region of the MS59-gene (SEQ ID NO:15) or the WL64-gene (SEQ ID NO: 17), respectively. These genes encode a hexose oxidase which is found to be toxic to (fungal) pathogens, and they are disclosed in WO 98/13478, which is hereby inclosed for reference.
15 It has been found that upon pathogen infection these genes are highly expressed, indicating pathogen inducibility. Pathogen inducible promoters are of great value in biotechnological resistance engineering.

Although the invention is exemplified especially with respect to
20 the promoter driving the expression of the hexose oxidase in sunflower, denoted as 'ms59', it is believed that all promoters driving genes homologous to this ms59 will have properties that are more or less identical to the promoter of ms59, i.e. expression activity which is induced by pathogen infection, preferably by fungal
25 infection. It is commonly known that promoters of homologous genes share similar properties, especially related to induction by pathogens. Examples of these are the promoters driving expression of the pathogenesis related protein osmotin, of which in potato (Zhu et al., Plant Physiol. 108, 929-937, 1995), in tobacco (Liu et al., Plant
30 Mol. Biol. 29, 1015-1026, 1995) and in tomato (Ruiz-Medrano et al., Plant Mol. Biol. 20, 1199-1202, 1992) the inducibility by pathogens has been established; and promoters driving the expression of genes of the PR-10 group, of which the pSTH-2 promoter in potato (Matton, D.P. and Brisson, N, Mol. Plant-Microbe Interac. 2, 325-331, 1989), the
35 AoPR-1 promoter in *Asparagus officinalis* (Warner, et al., The Plant J. 3, 191-201, 1993) and the Bet v 1 promoter in *Betula verrucosa* (Swoboda et al., Plant, Cell, Environm. 18, 865-874, 1995) have been shown to be pathogen inducible.

In this description the terms 'regulatory sequence' and 'promoter' are used interchangeably.

5 The present invention provides amongst others chimeric DNA sequences which comprise the regulatory sequences according to the invention. The expression chimeric DNA sequence shall mean to comprise any DNA sequence which comprises DNA sequences not naturally found in nature. For instance, chimeric DNA shall mean to comprise DNA comprising the regulatory region which is pathogen-inducible in a non-natural location of the plant genome, notwithstanding the fact that
10 said plant genome normally contains a copy of the said regulatory region in its natural chromosomal location. Similarly, the said regulatory region may be incorporated in the plant genome wherein it is not naturally found, or in a replicon or vector where it is not naturally found, such as a bacterial plasmid or a viral vector.
15 Chimeric DNA shall not be limited to DNA molecules which are replicable in a host, but shall also mean to comprise DNA capable of being ligated into a replicon, for instance by virtue of specific adaptor sequences, physically linked to the regulatory region according to the invention. The regulatory region may or may not be
20 linked to its natural downstream open reading frame.

The open reading frame of the gene which expression is driven by the pathogen-inducible regulatory regions of the invention may be derived from a genomic library. In this latter it may contain one or more introns separating the exons making up the open reading frame
25 that encodes a protein according to the invention. The open reading frame may also be encoded by one uninterrupted exon, or by a cDNA to the mRNA encoding a protein according to the invention. Chimeric DNA sequences according to the invention also comprise those in which one or more introns have been artificially removed or added. Each of these
30 variants is embraced by the present invention.

In order to be capable of being expressed in a host cell a regulatory region according to the invention will usually be provided with a transcriptional initiation region which may be suitably derived from any gene capable of being expressed in the host cell of choice,
35 as well as a translational initiation region for ribosome recognition and attachment. In eukaryotic cells, an expression cassette usually comprises in addition a transcriptional termination region located downstream of said open reading frame, allowing transcription to

terminate and polyadenylation of the primary transcript to occur. In addition, the codon usage may be adapted to accepted codon usage of the host of choice. Further, often a signal sequence may be encoded, which is responsible for the targeting of the gene expression product to subcellular compartments. The principles governing the expression of a chimeric DNA construct in a chosen host cell are commonly understood by those of ordinary skill in the art and the construction of expressible chimeric DNA constructs is now routine for any sort of host cell, be it prokaryotic or eukaryotic.

In order for the chimeric DNA sequence to be maintained in a host cell it will usually be provided in the form of a replicon comprising said chimeric DNA sequence according to the invention linked to DNA which is recognised and replicated by the chosen host cell. Accordingly, the selection of the replicon is determined largely by the host cell of choice. Such principles as govern the selection of suitable replicons for a particular chosen host are well within the realm of the ordinary skilled person in the art.

A special type of replicon is one capable of transferring itself, or a part thereof, to another host cell, such as a plant cell, thereby co-transferring the open reading frame according to the invention to said plant cell. Replicons with such capability are herein referred to as vectors. An example of such vector is a Ti-plasmid vector which, when present in a suitable host, such as *Agrobacterium tumefaciens*, is capable of transferring part of itself, the so-called T-region, to a plant cell. Different types of Ti-plasmid vectors (vide: EP 0 116 718 B1) are now routinely being used to transfer chimeric DNA sequences into plant cells, or protoplasts, from which new plants may be generated which stably incorporate said chimeric DNA in their genomes. A particularly preferred form of Ti-plasmid vectors are the so-called binary vectors as claimed in (EP 0 120 516 B1 and US 4,940,838). Other suitable vectors, which may be used to introduce DNA according to the invention into a plant host, may be selected from the viral vectors, e.g. non-integrative plant viral vectors, such as derivable from the double stranded plant viruses (e.g. CaMV) and single stranded viruses, gemini viruses and the like. The use of such vectors may be advantageous, particularly when it is difficult to stably transform the plant host. Such may be the case with woody species, especially trees and vines.

The expression "host cells incorporating a chimeric DNA sequence according to the invention in their genome" shall mean to comprise cells, as well as multicellular organisms comprising such cells, or essentially consisting of such cells, which stably incorporate said chimeric DNA into their genome thereby maintaining the chimeric DNA, and preferably transmitting a copy of such chimeric DNA to progeny cells, be it through mitosis or meiosis. According to a preferred embodiment of the invention plants are provided, which essentially consist of cells which incorporate one or more copies of said chimeric DNA into their genome, and which are capable of transmitting a copy or copies to their progeny, preferably in a Mendelian fashion. By virtue of the transcription and translation of the chimeric DNA according to the invention in some or all of the plant's cells, those cells that comprise said regulatory region will respond to pathogen attack and thus produce the protein encoded by the open reading frame which is under control of the regulatory region. In specific embodiments of the invention this protein will be an antipathogenic protein which is capable of conferring resistance to pathogen infections.

As is well known to those of skill in the art, regulatory regions of plant genes consist of distinct subregions with interesting properties in terms of gene expression. Examples of subregions as meant here, are enhancers but also silencers of transcription. These elements may work in a general (constitutive) way, or in a tissue-specific manner. Deletions may be made in the regulatory DNA sequences according to the invention, and the subfragments may be tested for expression patterns of the associated DNA. Various subfragments so obtained, or even combinations thereof, may be useful in methods of engineering pathogen resistance, or other applications involving the expression of heterologous DNA in plants. The use of DNA sequences according to the invention to identify functional subregions, and the subsequent use thereof to promote or suppress gene expression in plants is also encompassed by the present invention.

As regards the necessity of a transcriptional terminator region, it is generally believed that such a region enhances the reliability as well as the efficiency of transcription in plant cells. Use thereof is therefore strongly preferred in the context of the present invention.

Examples of proteins that may be used in combination with the ICS regulatory region according to the invention include, but are not limited to, β -1,3-glucanases and chitinases which are obtainable from
5 barley (Swegle M. et al., Plant Mol. Biol. 12, 403-412, 1989; Balance G.M. et al., Can. J. Plant Sci. 56, 459-466, 1976 ; Hoj P.B. et al., FEBS Lett. 230, 67-71, 1988; Hoj P.B. et al., Plant Mol. Biol. 13, 31-42, 1989), bean (Boller T. et al., Planta 157, 22-31, 1983; Broglie K.E. et al., Proc. Natl. Acad. Sci. USA 83, 6820-6824, 1986; Vögeli U.
10 et al., Planta 174, 364-372, 1988); Mauch F. & Staehelin L.A., Plant Cell 1, 447-457, 1989); cucumber (Metraux J.P. & Boller T., Physiol. Mol. Plant Pathol. 28, 161-169, 1986); leek (Spanu P. et al., Planta 177, 447-455, 1989); maize (Nasser W. et al., Plant Mol. Biol. 11, 529-538, 1988), oat (Fink W. et al., Plant Physiol. 88, 270-275,
15 1988), pea (Mauch F. et al., Plant Physiol. 76, 607-611, 1984; Mauch F. et al., Plant Physiol. 87, 325-333, 1988), poplar (Parsons, T.J. et al., Proc. Natl. Acad. Sci. USA 86, 7895-7899, 1989), potato (Gaynor J.J., Nucl. Acids Res. 16, 5210, 1988; Kombrink E. et al., Proc. Natl. Acad. Sci. USA 85, 782-786, 1988; Laflamme D. and Roxby R., Plant Mol.
20 Biol. 13, 249-250, 1989), tobacco (e.g. Legrand M. et al., Proc. Natl. Acad. Sci. USA 84, 6750-6754, 1987; Shinshi H. et al. Proc. Natl. Acad. Sci. USA 84, 89-93, 1987), tomato (Joosten M.H.A. & De Wit P.J.G.M., Plant Physiol. 89, 945-951, 1989), wheat (Molano J. et al., J. Biol. Chem. 254, 4901-4907, 1979), magainins, lectins, toxins
25 isolated from *Bacillus thuringiensis*, antifungal proteins isolated from *Mirabilis jalapa* (EP 0 576 483) and *Amaranthus* (EP 0 593 501 and US 5,514,779), albumin-type proteins (such as thionine, napin, barley trypsin inhibitor, cereal gliadin and wheat-alpha-amylase, EP 0 602 098), proteins isolated from *Raphanus*, *Brassica*, *Sinapis*, *Arabidopsis*,
30 *Dahlia*, *Cnicus*, *Lathyrus* and *Clitoria* (EP 0 603 216), proteins isolated from *Capsicum*, *Briza*, *Delphinium*, *Catapodium*, *Baptisia* and *Microsensis* (PCT/GB93/02179), oxalate oxidase (EP 0 636 181 and EP 0 673 416), saccharide oxidase (PCT/EP 97/04923), antimicrobial proteins isolated from *Allium* seeds (PCT/GB94/01636), proteins from *Aralia* and
35 *Impatiens* (PCT/GB95/00509), proteins from *Heuchera* and *Aesculus* (PCT/GB94/02766), mutant peptides to the above mentioned proteins (PCT/GB96/03065 and PCT/GB96/03068) and the like.

Another use of the inducible promoter is to drive proteins which play a role in the gene-for-gene resistance interaction (e.g. as described in WO 91/15585). Such proteins are, for example, plant proteins such as disclosed in Karrer, E.E. et al. (Plant Mol. Biol. 36, 681-690, 1998), activated ndr1, activated eds1 and activated Xa21, Cf-proteins, BS3 protein and Pto proteins from tomato, Rpm1 and Rps2 proteins from *Arabidopsis thaliana*, the N-gene from tobacco, the avr-elicitor proteins from *Cladosporium fulvum*, avrBs3 from *Xanthomonas*, harpins from *Erwinia* and the avrPto protein from *Pseudomonas*,

The actual applicability of the invention is not limited to certain plant species. Any plant species that is subject to some form of pathogen attack, may be transformed with chimeric DNA sequences according to the invention, allowing the regulatory region to be induced by pathogen infection thereby triggering production of antipathogenic proteins to be produced in some or all of the plant's cells.

Although some of the embodiments of the invention may not be practicable at present, e.g. because some plant species are as yet recalcitrant to genetic transformation, the practicing of the invention in such plant species is merely a matter of time and not a matter of principle, because the amenability to genetic transformation as such is of no relevance to the underlying embodiment of the invention.

Transformation of plant species is now routine for an impressive number of plant species, including both the *Dicotyledoneae* as well as the *Monocotyledoneae*. In principle any transformation method may be used to introduce chimeric DNA according to the invention into a suitable ancestor cell, as long as the cells are capable of being regenerated into whole plants. Methods may suitably be selected from the calcium/polyethylene glycol method for protoplasts (Krens, F.A. et al., Nature 296, 72-74, 1982; Negrutiu I. et al., Plant Mol. Biol. 8, 363-373, 1987), electroporation of protoplasts (Shillito R.D. et al., Bio/Technol. 3, 1099-1102, 1985), microinjection into plant material (Crossway A. et al., Mol. Gen. Genet. 202, 179-185, 1986), DNA (or RNA-coated) particle bombardment of various plant material (Klein T.M. et al., Nature 327, 70, 1987), infection with (non-integrative) viruses and the like. A preferred method according to the invention

comprises *Agrobacterium*-mediated DNA transfer. Especially preferred is the use of the so-called binary vector technology as disclosed in EP A 120 516 and U.S. Patent 4,940,838.

Tomato transformation is preferably done essentially as described by Van Roekel et al. (Plant Cell Rep. 12, 644-647, 1993). Potato transformation is preferably done essentially as described by Hoekema et al. (Hoekema, A. et al., Bio/Technology 7, 273-278, 1989). Generally, after transformation plant cells or cell groupings are selected for the presence of one or more markers which are encoded by plant expressible genes co-transferred with the nucleic acid sequence encoding the protein according to the invention, whereafter the transformed material is regenerated into a whole plant.

Although considered somewhat more recalcitrant towards genetic transformation, monocotyledonous plants are amenable to transformation and fertile transgenic plants can be regenerated from transformed cells or embryos, or other plant material. Presently, preferred methods for transformation of monocots are microprojectile bombardment of embryos, explants or suspension cells, and direct DNA uptake or electroporation (Shimamoto, et al, Nature 338, 274-276, 1989).

Transgenic maize plants have been obtained by introducing the *Streptomyces hygroscopicus* bar-gene, which encodes phosphinothricin acetyltransferase (an enzyme which inactivates the herbicide phosphinothricin), into embryogenic cells of a maize suspension culture by microprojectile bombardment (Gordon-Kamm,, Plant Cell, 2, 603-618, 1990). The introduction of genetic material into aleurone protoplasts of other monocot crops such as wheat and barley has been reported (Lee, Plant Mol. Biol. 13, 21-30, 1989). Wheat plants have been regenerated from embryogenic suspension culture by selecting only the aged compact and nodular embryogenic callus tissues for the establishment of the embryogenic suspension cultures (Vasil, Bio/Technol. 8, 429-434, 1990). The combination with transformation systems for these crops enables the application of the present invention to monocots.

Monocotyledonous plants, including commercially important crops such as rice and corn are also amenable to DNA transfer by *Agrobacterium* strains (vide WO 94/00977; EP 0 159 418 B1; Gould J, et al., Plant. Physiol. 95, 426-434, 1991).

Following DNA transfer and regeneration, putatively transformed plants may be evaluated, for instance using Southern analysis, for the presence of the chimeric DNA according to the invention, copy number and/or genomic organization. In addition, or alternatively, expression levels of the newly introduced DNA may be undertaken, using Northern and/or Western analysis, techniques well known to persons having ordinary skill in the art. After the initial analysis, which is optional, transformed plants showing the desired copy number and expression level of the newly introduced chimeric DNA according to the invention may be tested for resistance levels against pathogens.. Alternatively, the selected plants may be subjected to another round of transformation, for instance to introduce further genes, in order to enhance resistance levels, or broaden the resistance.

Other evaluations may include the testing of pathogen resistance under field conditions, checking fertility, yield, and other characteristics. Such testing is now routinely performed by persons having ordinary skill in the art.

Following such evaluations, the transformed plants may be grown directly, but usually they may be used as parental lines in the breeding of new varieties or in the creation of hybrids and the like.

To obtain transgenic plants capable of constitutively expressing more than one chimeric gene, a number of alternatives are available including the following:

A. The use of DNA, e.g. a T-DNA on a binary plasmid, with a number of modified genes physically coupled to a selectable marker gene. The advantage of this method is that the chimeric genes are physically coupled and therefore migrate as a single Mendelian locus.

B. Cross-pollination of transgenic plants each already capable of expressing one or more chimeric genes, preferably coupled to a selectable marker gene, with pollen from a transgenic plant which contains one or more chimeric genes coupled to another selectable marker. Afterwards the seed, which is obtained by this crossing, maybe selected on the basis of the presence of the two selectable markers, or on the basis of the presence of the chimeric genes themselves. The plants obtained from the selected seeds can afterwards be used for further crossing. In principle the chimeric genes are not on a single locus and the genes may therefore segregate as independent loci.

C. The use of a number of a plurality chimeric DNA molecules, e.g. plasmids, each having one or more chimeric genes and a selectable marker. If the frequency of co-transformation is high, then selection on the basis of only one marker is sufficient. In other cases, the selection on the basis of more than one marker is preferred.

D. Consecutive transformation of transgenic plants already containing a first, second, (etc), chimeric gene with new chimeric DNA, optionally comprising a selectable marker gene. As in method B, the chimeric genes are in principle not on a single locus and the chimeric genes may therefore segregate as independent loci.

E. Combinations of the above mentioned strategies.

The actual strategy may depend on several considerations as maybe easily determined such as the purpose of the parental lines (direct growing, use in a breeding programme, use to produce hybrids) but is not critical with respect to the described invention.

In this context it should be emphasised that plants already containing chimeric DNA may form a suitable genetic background for introducing further chimeric DNAs according to the invention, for instance in order to enhance the production antipathogenic substances, thereby enhancing resistance levels. The cloning of other genes corresponding to proteins that can suitably be used in combination with the regulatory DNA fragments, and the obtention of transgenic plants, capable of relatively over-expressing same, as well as the assessment of their effect on pathogen resistance in planta, is now within the scope of the ordinary skilled person in the art.

Plants with improved resistance against pathogens may be grown in the field, in the greenhouse, or at home or elsewhere. Plants or edible parts thereof may be used for animal feed or human consumption, or may be processed for food, feed or other purposes in any form of agriculture or industry. Agriculture shall mean to include horticulture, arboriculture, flower culture, and the like. Industries which may benefit from plant material according to the invention include but are not limited to the pharmaceutical industry, the paper and pulp manufacturing industry, sugar manufacturing industry, feed and food industry, enzyme manufacturers and the like. The advantages of the plants, or parts thereof, according to the invention are the decreased need for biocide treatment, thus lowering

costs of material, labour, and environmental pollution, or prolonging shelf-life of products (e.g. fruit, seed, and the like) of such plants. Plants for the purpose of this invention shall mean multicellular organisms capable of photosynthesis, and subject to some form of pathogen attack. They shall at least include angiosperms as well as gymnosperms, monocotyledonous as well as dicotyledonous plants.

EXAMPLE 1

Induction of ms59 messengers in sunflower plants.

Leaves of 7 to 8 weeks old Sunflower plants (*Helianthus annuus* cv Zebulon) were induced by spraying 5 times with 5 mM salicylic acid (SA), spraying once with 1mM salicylic acid, once with 0.1 mM jasmonic acid (JA), once with 1 mM ACC (1-aminocyclopropane-1-carboxylic acid, a precursor of the plant hormone ethylene) or wounding. Leaf samples were harvested from induced leaves after 24 hours (1 mM SA, 0.1 mM JA, 1 mM ACC and wounding) and after 5 days (5 mM SA). Control samples were taken at 24 hours after induction in non-induced plants.

EXAMPLE 2

RNA extraction from sunflower leaf tissue and cDNA synthesis.

Total RNA was extracted from 10 g leaf material using a hot phenol method and purified using the Qiagen RNA buffer set and tip-100 columns (Qiagen GmbH, Germany). Contaminating DNA was degraded using a Dnase I (Gibco BRL) treatment.

cDNA was prepared using 1 µg total RNA, 1 µl oligo(dT)₁₂₋₁₈ primers (500 µg/ml, Gibco BRL) and 200 units of Superscript II RT RNase H⁻ reverse transcriptase (Gibco BRL) as described by the manufacturer.

EXAMPLE 3

Construction of ms59 PCR MIMIC and analysis of samples by competitive RT-PCR.

Transcript levels of ms59 were determined using the competitive RT-PCR technology. In this technique competition between the cDNA target and an artificial PCR MIMIC makes quantitation of transcript levels

possible (Paul D. Siebert and James W. Larrick (1992), Nature 359, 557-558).

For construction of a PCR MIMIC the following primers were developed; FR-pUC-208 (SEQ ID NO: 1) 5' GTT CCG GAG GTT GTG ACC GTG GGA TGT GCT GCA AGG CG3', FR-pUC-209 (SEQ ID NO: 2) 5' CTG GGG AAG CCC GTG TAG TAA AGC CCC CGC GCG TTG GCC GAT TC3', FR-MS59-47 (SEQ ID NO: 3) 5' CTG GGG AAG CCC GTG TAG TAA AGC3' and FR-MS59-77 (SEQ ID NO: 4) 5' GTT CCG GAG GTT GTG ACC GTG3'. Primers FR-pUC-208 and FR-pUC-209 were used to amplify a fragment of 387 bp from the plasmid pUC18 (Yanisch-Perron, C., Vieira, J. and Messing, J. (1985) Gene 33, 103-119) by PCR (10 cycles of 1' 95°C, 1' 55°C, 2' 72°C). From this PCR product 1 µl was amplified using primers FR-MS59-47 and FR-MS59-77 by PCR to produce a large amount of PCR MIMIC (30 cycles of 1' 95°C, 1' 55°C, 2' 72°C).

Primers FR-MS59-47 and FR-MS59-77 will amplify a band of 312 bp from the *ms59* cDNA so it can be distinguished easily from the 387 bp MIMIC when separated on a 2% agarose gel. PCR MIMIC dilutions were made in a range of 100 ng/µl to 0.01 ag/µl in H₂O containing 0.2 µg/µl glycogen as a carrier.

Table 1: Induction levels of the *ms59* messenger in sunflower leaves after different stress treatments relative to the control.

Induction method	fold induction
Control	1 ^a
5 mM salicylic acid	1000
1 mM salicylic acid	1 ^a
1 mM ACC	10
0.1 mM jasmonic acid	10
wounding	10

note: ^a: could not be detected, arbitrary set to 1.

The cDNA samples were analysed in a competitive RT-PCR. Therefore 2 µl of the samples was combined in a 0.5 ml tube with 1 µl diluted PCR MIMIC (amounts; 0.1 pg, 10 fg, 1.0 fg, 0.1 fg, 10 ag and 1.0 ag).

Amplification of cDNA and MIMIC was performed using 10 μ M of the primers FR-MS59-47 and FR-MS59-77, 0.5 μ l 20 mM dNTP's, 1x PCR buffer, $MgCl_2$ and 2.5 units recombinant Taq DNA polymerase (Gibco BRL) and was allowed to proceed for 35 cycles, 1' 95°C, 1' 55°C, 2' 72°C. PCR products were separated on a 2% agarose gel and visualized by staining with ethidium bromide and a UV illuminator.

EXAMPLE 4

Infection assays in sunflower plants with different fungi.

Fungal infections were performed on plants of 7 to 8 weeks old. Leaves were inoculated by laying small droplets (15-20 μ l) of a *Botrytis cinerea* spore suspension, *Diaporthe helianthi* (PH9905) hyphal fragment suspension or a *Sclerotinia sclerotiorum* hyphal fragment suspension on small cuttings made in the leaf to enable the fungi to penetrate the plant. Fungal infections were allowed to proceed at 18°C and a high relative humidity (\pm 90%). Leaf disks (diameter = 13 mm) harbouring the site of infection were harvested at approximately 4 days after inoculation. Around the hole of the first small leaf disk, a further ring of 25 mm was harvested. Leaf disks were also harvested in non-infected leaves around leaf cuttings as a control.

EXAMPLE 5

PolyA⁺-RNA extraction from sunflower leaf tissue and cDNA synthesis.

Poly-A⁺ RNA was harvested from 100 mg of leaf tissue using the Quickprep Micro mRNA purification Kit (Amersham Pharmacia Biotech, Uppsala, Sweden). The relative amount of mRNA was determined using visualisation of nucleic acids by spotting 10 μ l of the samples with 4 μ l 1 μ g/ml ethidium bromide on a UV illuminator.

Equal amounts of Poly-A⁺ RNA (\pm 100 ng) were used to synthesize cDNA using 200 units of Superscript II RT RNase H⁻ reverse transcriptase (Gibco BRL) and 1 μ l oligo(dT)₁₂₋₁₈ primers (500 μ g/ml, Gibco BRL) as described by the manufacturer.

Example 6

Analysis of samples by competitive RT-PCR.

The different cDNA samples were analysed as described in example 3.

Table 2: Induction levels of the ms59 messenger after infection of sunflower leaves with different fungi.

Sample	fold induction leaf disk ^b	fold induction outer infection area ^c
Control	1 ^a	1 ^a
<i>Diaporthe helianthi</i>	1000	1000
<i>Botrytis cinerea</i>	1000	1 ^a
<i>Sclerotinia sclerotiorum</i>	1000	1 ^a

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note: ^a: arbitrary set to 1.

^b: leaf disk of 13 mm around site of fungal infection.

^c: leaf ring from 13 mm to 25 mm around site of fungal infection.

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EXAMPLE 7

Construction of a sunflower gapC PCR MIMIC and analysis of samples by competitive RT-PCR.

As an internal control on the quality of the mRNA and preparation of cDNA we included a competitive RT-PCR on the housekeeping gene GapC using the same samples as described in examples 4, 5 and 6.

For the construction of a PCR MIMIC the following primers were developed; FR-pUC-224 (SEQ ID NO: 5) 5' CCA TGG GCT CAA ACT GGA GCC GGC CGG GAG CAG ACA AGC CCG 3', FR-pUC-225 (SEQ ID NO: 6) 5' CGA GAC GTC AAC AGT CGG GAC CCA CTC ATT AGG CAC CCC AGG C3', FR-gapC-211 (SEQ ID NO: 7) 5' CCA TGG GCT CAA ACT GGA GCC G3' and FR-gapC-212 (SEQ ID NO: 8) 5' CGA GAC GTC AAC AGT CGG GAC C3'. Primers FR-pUC-224 and FR-pUC-225 were used to amplify a fragment of 527 bp from the plasmid

pUC18 (Yanisch-Perron, C., Vieira, J. and Messing, J. (1985) Gene 33, 103-119) by PCR (10 cycles of 1' 95°C, 1' 55°C, 2' 72°C). From this PCR product 1 µl was amplified using primers FR-gapC-211 and FR-gapC-212 by PCR to produce a large amount of PCR MIMIC (30 cycles of 1' 95°C, 1' 55°C, 2' 72°C).

Primers FR-gapC-211 and FR-gapC-212 will amplify a band of 470 bp from the gapC cDNA so it can be distinguished easily from the 527 bp MIMIC when separated on a 2% agarose gel. PCR MIMIC dilutions were made in a range of 100 ng/µl to 0.01 ag/µl in H₂O containing 0.2 µg/µl glycogen as a carrier.

The cDNA samples were analysed in a competitive RT-PCR. Therefore 2 µl of each sample was combined in a 0.5 ml tube with 1 µl diluted PCR MIMIC (amounts; 0.1 pg, 10 fg, 1.0 fg, 0.1 fg, 10 ag and 1.0 ag).

Amplification of cDNA and MIMIC was performed using 10 µM of the primers FR-gapC-211 and FR-gapC-212, 0.5 µl 20 mM dNTP's, 1x PCR buffer, MgCl₂ and 2.5 units recombinant Taq DNA polymerase (Gibco BRL) and was allowed to proceed for 35 cycles. 1' 95°C, 1' 55°C, 2' 72°C. PCR products were separated on a 2% agarose gel and visualized by staining with ethidium bromide and a UV illuminator.

Table 3: Induction levels of the control GapC messenger after infection of sunflower leaves with different fungi.

Sample	fold induction in 13 mm leaf disks
Control	1 ^a
<i>Diaporthe helianthi</i>	10
<i>Botrytis cinerea</i>	10
<i>Sclerotinia sclerotiorum</i>	10

note: ^a: arbitrary set to 1.

The results show that RNA quality and cDNA preparation is not affected by these fungal infections. Induction of GapC messengers by plant pathogens and environmental stress factors was described before in Laxalt et al., (1996) Plant Mol. Biol. 30: 961-972.

EXAMPLE 8

Isolation of the ms59 promoter from the sunflower genome.

For the isolation of the ms59 promoter genomic DNA was isolated from sunflower leaves using a CTAB extraction procedure. About 10 µg of genomic DNA was digested with the restriction enzymes *BspH* I, *EcoR* V, *Nla* IV, *Hph* I, *Rsa* I, *Ssp* I and *Hind* III for 16 hours at 37°C. These restriction sites are all located within the first part of the ms59 cDNA. The digestion mixtures were extracted with 1 volume phenol:chloroform:isoamylalcohol (25:24:1, v/v, Gibco BRL) and precipitated with 0.1 volume of 3 M NaAc (pH=5.2) and 2.5 volumes of 96% ethanol. The DNA pellet was washed with 70% ethanol and the pellet was then dissolved in 50 µl distilled water. 25 µl of each sample was separated on a 0.7% agarose gel for 16 hours at 40 volts. The DNA was transferred to a nylon membrane (Hybond-N⁺, Amersham Life Science) using southern blotting with 0.4 M NaOH. The blot was hybridized (16 hours, 65°C) using a 320 bp fragment (from the ATG startcodon until the *BspH* I site) labeled with ³²P-dCTP as a probe. Then the blot was washed with a stringency of 0.2x SSC at 65°C. The results of the southern blot are listed in table 4.

The remaining 25 µl of digestion mixture was ligated in such a way that circularization of the DNA fragments was stimulated. This was done by ligating the DNA in a (large) volume of 300 µl in 1x T4 ligation buffer and 5 weiss units of T4 DNA ligase (Gibco BRL) for 16 hours at 16°C. Again the mixture was extracted with phenol:chloroform:isoamylalcohol and precipitated with ethanol and the DNA pellet was dissolved in 50 µl H₂O.

Primers were designed within the first part of the cDNA (between the ATG startcodon and the first restriction site used (*BspH* I) and

directed outwards. Primers FR-MS59-11 (SEQ ID NO: 9) 5' CAG GCA GCT GTG GTT TGT GGC3' and FR-MS59-49 (SEQ ID NO: 10) 5' CGG GAA GTT GCA GAA GAT TGG GTT G3' were used in a PCR reaction on 1 μ l of the ligation mixture using the Advantage KlenTaq polymerase mix (Clontech laboratories, Inc., Palo Alto, CA) 200 μ M dNTP's and 10 μ M of each primer. The polymerase mix was activated for 1' at 94°C followed by 35 cycles of amplification for 30" at 94°C, 1' at 55°C and 3' at 68°C. The PCR products were analysed on a 1% agarose gel and no specific band could be detected. Therefore nested PCR was performed as described above but now with nested primers FR-MS59-34 (SEQ ID NO: 11) 5' ACG TAG ATA TCG AAC AAG AAA CCG C3' and FR-MS59-50 (SEQ ID NO: 12) 5' GAG CAA GAG AAG AAG GAG AC3' using 1 μ l of the PCR product from the first PCR round. After analysis of the PCR products on a 1% agarose gel very specific single bands were detected. Inverse PCR results are listed in table 4.

Table 4: Results inverse PCR and southern blot band sizes (nd= not determined)

Inverse PCR restriction enzyme	Band sizes southern blot	Band sizes nested PCR
<i>Bsp</i> H I	> 7 kb	nd
<i>Eco</i> R V	> 7 kb	nd
<i>Nla</i> IV	1.0 kb	0.6 kb
<i>Hph</i> I	1.2 kb	0.8 kb
<i>Rsa</i> I	0.5 kb	0.2 kb
<i>Ssp</i> I	1.3 kb	0.9 kb
<i>Hind</i> III	2.3 kb	1.9 kb

The 1.9 kb *Hind* III iPCR band was isolated from gel and the DNA sequence of the ends was determined using primers FR-MS59-34 and FR-MS59-50 on a automatic DNA sequencer (Applied Biosystems).

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Based on the DNA sequence new primers were designed for the amplification of the *ms59* promoter region from the sunflower genome. Primer FR-MS59-226 (SEQ ID NO: 13) 5' GCA AGC TTT ATA GTT TAC GAT CC3' is directed downstream, located in the upstream part of the *ms59* promoter region overlapping the *Hind* III restriction site. Primer FR-MS59-227 (SEQ ID NO: 14) 5' TTG CCA TGG TGC ATG GTT TAG CG3' can anneal at the most downstream part of the *ms59* promoter/leader overlapping the ATG translational start introducing a *Nco* I restriction site spanning the ATG startcodon. The DNA sequence of the complete promoter fragment from the upstream *Hind* III to *Nco* I site (SEQ ID NO: 15, nucleotides 1-1889) was determined using automated DNA sequence analysis (Applied Biosystems).

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Using *Pfu* DNA polymerase (Stratagene) and both primers the *ms59* promoter region was amplified from sunflower genomic DNA. The 1.9 kb PCR product was digested with *Hind* III and *Nco* I and ligated in a multicopy cloning vector also digested with *Hind* III and *Nco* I. The promoter was fused to the GUSintron reporter gene (Jefferson et al., (1987) EMBO J 6: 3901-3907) followed by the 3' untranslated region of the potato proteinase inhibitor II gene (Thornburg et al., 1987, Proc. Natl. Acad. Sci. USA 84, 744-748) which contains sequences needed for polyadenylation (An et al., 1989, Plant Cell 1, 115-122) using restriction sites *Nco* I and *Eco*R I resulting in pMOG1367. The entire chimeric gene flanked by restriction sites *Eco*R I and *Hind* III was then transferred to pMOG800 (for description of this plasmid see for example WO 97/42326) digested with *Eco*R I and *Hind* III.

The resulting binary vector pMOG1368 was introduced in *Agrobacterium tumefaciens* strain EHA105 for transformation of target crops potato and tomato, strain MOG101 for transformation of tobacco and *Arabidopsis thaliana* and strain MOG301 for transformation of *Brassica napus*.

Example 9

Transformation of pMOG1368 to potato cv Kardal.

pMOG 1368 was transformed to potato essentially as described by
5 Hoekema et al. (Hoekema, A. et al., Bio/Technology 7, 273-278, 1989).
In short, potatoes (*Solanum tuberosum* cv. Kardal) were transformed
with the *Agrobacterium* strain EHA 105 pMOG 1368. The basic culture
medium was MS30R3 medium consisting of MS salts (Murashige and Skoog
10 (1962) *Physiol. Plant.* 14, 473), R3 vitamins (Ooms et al. (1987)
Theor. Appl. Genet. 73, 744), 30 g/l sucrose, 0.5 g/l MES with final
pH 5.8 (adjusted with KOH) solidified when necessary with 8 g/l
Daichin agar. Tubers of *Solanum tuberosum* cv. Kardal were peeled and
surface sterilized by burning them in 96% ethanol for 5 seconds. The
15 flames were extinguished in sterile water and cut slices of
approximately 2 mm thickness. Disks were cut with a bore from the
vascular tissue and incubated for 20 minutes in MS30R3 medium
containing $1-5 \times 10^8$ bacteria/ml of *Agrobacterium* EHA 105 containing
the binary vector. The tuber discs were washed with MS30R3 medium and
20 transferred to solidified postculture medium (PM). PM consisted of
M30R3 medium supplemented with 3.5 mg/l zeatin riboside and 0.03 mg/l
indole acetic acid (IAA). After two days, discs were transferred to
fresh PM medium with 200 mg/l cefotaxim and 100 mg/l vancomycin. Three
days later, the tuber discs were transferred to shoot induction medium
(SIM) which consisted of PM medium with 250 mg/l carbenicillin and 100
25 mg/l kanamycin. After 4-8 weeks, shoots emerging from the discs were
excised and placed on rooting medium (MS30R3-medium with 100 mg/l
cefotaxim, 50 mg/l vancomycin and 50 mg/l kanamycin). The shoots were
propagated axenically by meristem cuttings.

Example 10

Testing of promoter function in transgenic potato plants

Transgenic potato plants harbouring the pMOG1368 ms59 promoter-GUS
construct were grown in tubes in vitro and assayed for expression of
35 the GUS gene. For this purpose leaf, stem and root samples were taken
and stained (results in table 5). GUS expression levels were
determined visually, on a scale of 0 to 5, where 0 is no detectable
expression and 5 is the highest level of GUS we have observed in

leaves of a transgenic plant, of a rare tobacco 35S-GUS-transgenic (line 96306). Samples from leaves of this plant were included in all experiments for internal reference.

5 Table 5: Expression of the GUS gene driven by the ms59 promoter in leaves, stems and roots of small in vitro plantlets.

Plant number	Leaf	Stem	Root
1368-1	0	0	0
1368-2	0	0	0
1368-3	0	0	0
1368-4	0	0	0
1368-5	0	0	0
1368-6	0	0	0
1368-7	0	0	0
1368-8	0	0	0
1368-9	0	0	0
1368-10	0	0	0
1368-11	0	0	0
1368-12	0	0	1
1368-13	0	0	0
1368-14	0	0	0
1368-15	0	0	0
1368-16	0	0	0
1368-17	0	0	0
1368-18	0	0	1
1368-19	0	0	0
1368-20	0	0	0
1368-21	0	0	0
1368-22	0	0	0
1368-23	0	0	0
1368-24	0	1	0
1368-25	0	0	0
1368-26	0	0	0
1368-27	0	0	0
1368-28	0	0	0
1368-29	0	0	0
1368-30	0	0	0

A selection of in vitro plantlets of the same age were infected with the potato late blight causing fungus *Phytophthora infestans*. Small droplets of water containing a high concentration of fungal spores were applied on the leaf surface. The infection was left to proceed at room temperature for 96 hours. Leaves which showed disease symptoms were removed from the plantlets and stained for expression of the GUS gene by histochemical GUS analysis (Goddijn et al., The Plant Journal (1993) 4(5): 863-873). Expression was monitored in the lesion resulting from the fungal infection, in the primary zone (the area just around the site of infection) and in the uninfected part of the leaf (background).

Table 6: Expression of the GUS gene driven by the ms59 promoter in leaves of potato in vitro plantlets infected by *P. infestans*

Plant number	before infection	lesion	primary zone	background
1368-1	0	0	0	0
1368-3	0	0	0	0
1368-4	0	0	1	0
1368-5	0	0	1	0
1368-6	0	0	0	0
1368-7	0	0	0	0
1368-8	0	0	0	0
1368-9	0	0	1	0

Promoter performance was also tested in the leaves of full grown potato plants before and after infection with *P. infestans*. Before inoculation leaves were detached and stained for expression of GUS. The plants were then sprayed with a spore suspension of 5×10^5 spores/ml and the infection was allowed to develop for 4 days (96 hours). Again leaves were detached and stained for the expression of GUS. The results are listed in table 7. Expression was monitored in the lesion, primary zone and the background area.

Table 7: Expression of the GUS gene driven by the ms59 promoter in^a leaves of transgenic potato plants before and after infection with *P. infestans*.

Plant number	before infection	lesion	primary zone	background
1368-1	0	0	0	1
1368-2	0	0	0	1
1368-3	0	0	0	0
1368-4	0	0	0	0
1368-5	0	0	0	0
1368-6	0	0	0	0
1368-7	0	0	0	0
1368-9	0	0	0	0
1368-10	0	0	0	0
1368-11	0	0	0	0
1368-12	2	0	2	2
1368-13	0	0	0	0
1368-14	0	0	0	0
1368-15	0	0	0	0
1368-16	0	0	0	0
1368-17	0	0	0	0
1368-18	0	0	2	0
1368-19	0	0	0	0
1368-20	0	0	0	0
1368-21	0	0	0	0
1368-22	0	0	0	0
1368-23	0	0	0	0
1368-24	0	0	0	0
1368-25	0	0	0	0
1368-26	0	0	3	0

5

The results showed that the ms59 promoter responds to fungal infection. The level of induced expression is rather low and may be below the detection level in some cases. This would explain the low frequency of detectable inducible GUS expression.

Claims:

1. A DNA fragment naturally driving the expression of a plant gene coding for hexose oxidase, capable of promoting pathogen-inducible transcription of an associated DNA sequence when re-introduced into a plant.

2. A DNA fragment according to claim 1, characterised in that it is obtainable from *Helianthus annuus*.

3. A DNA fragment according to claim 1, characterised in that it is obtainable from *Lactuca sativa*.

4. A DNA fragment according to claim 2 characterized in that it is the upstream regulatory region of the gene coding for hexose oxidase, denoted as MS59.

5. A DNA fragment according to claim 3, characterized in that it is the upstream regulatory region of the gene coding for hexose oxidase, denoted as WL64.

6. A DNA fragment according to claim 4, characterized in that it comprises the nucleotide sequence from 1 to 1889 depicted in SEQ ID NO: 15.

7. A portion or variant of a DNA fragment according to any one of claims 4 to 6, capable of promoting pathogen-inducible transcription of an associated DNA sequence when re-introduced into a plant.

8. A chimeric DNA sequence comprising in the direction of transcription a DNA fragment according to any one of claims 1 to 7 and a DNA sequence to be expressed under the transcriptional control thereof and which is not naturally under transcriptional control of said DNA fragment.

9. A chimeric DNA sequence according to claim 8, wherein the DNA sequence to be expressed causes the production of an antipathogenic protein.

antipathogenic protein is selected from the group consisting of chitinase, glucanase, osmotin, magainins, lectins, saccharide oxidase, oxalate oxidase, toxins from *Bacillus thuringiensis*, antifungal proteins isolated from *Mirabilis jalapa*, *Amaranthus*, *Raphanus*, *Brassica*, *Sinapis*, *Arabidopsis*, *Dahlia*, *Cnicus*, *Lathyrus*, *Clitoria*, *Allium* seeds, *Aralia* and *Impatiens* and albumin-type proteins, such as thionine, napin, barley trypsin inhibitor, cereal gliadin and wheat-alpha-amylase.

11. A chimeric DNA sequence according to claim 8, wherein the DNA sequence to be expressed causes the production of a protein that can induce a hypersensitive response, preferably selected from the group consisting of Cf, Bs3 and Pto proteins from tomato, Rpm1 and Rps2 from *Arabidopsis thaliana*, N-protein from tobacco, avr proteins from *Cladosporium fulvum*, harpins from *Erwinia* and elicitor proteins (avrBs3, avrRpm1, avrRpt2) from *Pseudomonas* or *Xanthomonas*.

12. A replicon comprising a chimeric DNA sequence according to any one of claims 8 to 11.

13. A replicon comprising in the direction of transcription a DNA fragment according to any one of claims 1 to 7 and at least one recognition site for a restriction endonuclease for insertion of a DNA sequence to be expressed under the control of said DNA fragment.

14. A microorganism containing a replicon according to any one of claims 12 or 13.

15. A plant cell having incorporated into its genome a chimeric DNA sequence according to any one of claims 8 to 11.

16. A plant essentially consisting of cells according to claim 15.

17. A plant according to claim 16 which is a dicotyledonous plant.

18. A part of a plant selected from seeds, flowers, tubers, roots, leaves, fruits, pollen and wood, obtained from a plant according to claim 16 or 17.

identifying homologues capable of promoting pathogen-induced transcription in a plant.

5 20. Use of a chimeric DNA sequence according to any one of claims 8 to 11 for transforming plants.

21. Use of a portion or variant according to claim 7 for making hybrid regulatory DNA sequences.

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22. Use of a chimeric DNA sequence according to any of claims 9 to 11 for conferring pathogen resistance to a plant.

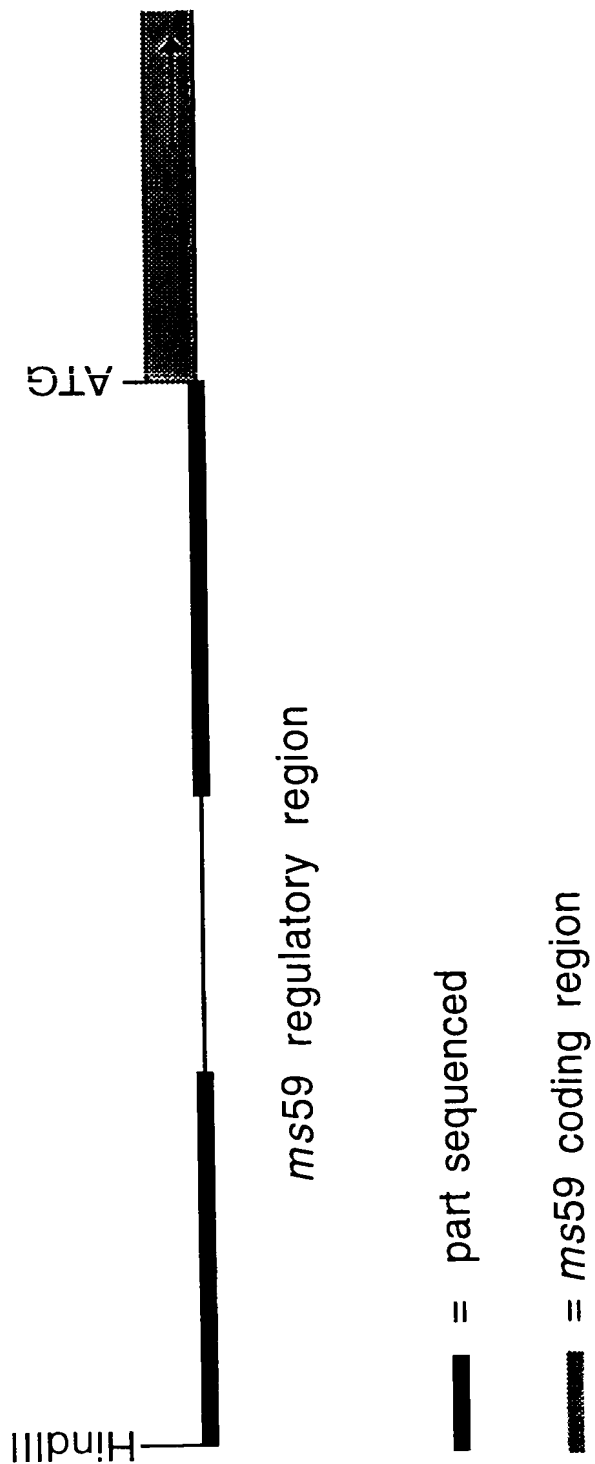


Fig. 1

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	aac atg caa	act tcc att	ctt act ctc	ctt ctt ctc	ttg ctc tca	acc	1961
35	Asn Met Gln	Thr Ser Ile	Leu Thr Leu	Leu Leu Leu	Leu Leu Leu	Ser Thr	
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	tta cac gac	cgg gcc gac	cct tca ttt	ccg ata acc	gga gag gtt	tac	2057
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	act ccc gga	aac tca tct	ttt cct acc	gtc ttg caa	aac tac atc	cga	2105
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55	Thr Ala Glu	His Val Ser	His Ile Gln	Ala Ala Val	Val Val Cys	Gly Lys	
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10	tgg gtc caa gcc ggt gcg act ctt ggt gaa gtg tac tat cga ata gcg	2393
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	Glu Lys Ser Asn Lys His Gly Phe Pro Ala Gly Val Cys Pro Thr Val	
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	Gly Val Gly Gly His Phe Ser Gly Gly Gly Tyr Gly Asn Leu Met Arg	
	185 190 195 200	
25	aaa tat ggt ttg tcg gtt gat aat att gtt gat gct caa ata ata gat	2537
	Lys Tyr Gly Leu Ser Val Asp Asn Ile Val Asp Ala Gln Ile Ile Asp	
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	Val Asn Gly Lys Leu Leu Asp Arg Lys Ser Met Gly Glu Asp Leu Phe	
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35	tgg gcg atc acc ggc ggt ggt ggt gtt agt ttt ggt gtg gtt cta gcc	2633
	Trp Ala Ile Thr Gly Gly Gly Val Ser Phe Gly Val Val Leu Ala	
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40	tac aaa atc aaa cta gtt cgt gtt ccg gag gtt gtg acc gtg ttt acc	2681
	Tyr Lys Ile Lys Leu Val Arg Val Pro Glu Val Val Thr Val Phe Thr	
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	Met Ser Trp Val Glu Ser Val Leu Tyr Tyr Thr Gly Phe Pro Ser Gly	
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5 ttc aag atc aaa tcc gat tat gtg caa aat cct att tct aaa cga cag 3065
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10 ttc gag ttc atc ttc gaa agg ctg aaa gaa ctt gaa aac caa atg ttg 3113
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15 gct ttc aac cca tat ggt ggt aga atg agt gaa ata tcc gaa ttc gca 3161
 Ala Phe Asn Pro Tyr Gly Gly Arg Met Ser Glu Ile Ser Glu Phe Ala
 410 415 420

20 aag cct ttc cca cat aga tcg ggt aac ata gcg aaa att caa tac gaa 3209
 Lys Pro Phe Pro His Arg Ser Gly Asn Ile Ala Lys Ile Gln Tyr Glu
 425 430 435 440

gta aac tgg gag gat ctt agc gat gaa gcc gaa aat cgt tac ttg aat 3257
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25 ttc aca agg ctg atg tat gat tac atg acc cca ttt ttg tcg aaa aac 3305
 Phe Thr Arg Leu Met Tyr Asp Tyr Met Thr Pro Phe Val Ser Lys Asn
 460 465 470

30 cct aga aaa gca ttt ttg aac tat agg gat ttg gat att ggt atc aac 3353
 Pro Arg Lys Ala Phe Leu Asn Tyr Arg Asp Leu Asp Ile Gly Ile Asn
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35 agc cat ggc agg aat gct tat act gaa gga atg gtt tat ggg cac aag 3401
 Ser His Gly Arg Asn Ala Tyr Thr Glu Gly Met Val Tyr Gly His Lys
 490 495 500

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 Tyr Phe Lys Glu Thr Asn Tyr Lys Arg Leu Val Ser Val Lys Thr Lys
 505 510 515 520

40 gtt gat cct gac aac ttc ttt agg aat gag caa agc atc cca act ttg 3497
 Val Asp Pro Asp Asn Phe Phe Arg Asn Glu Gln Ser Ile Pro Thr Leu
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45 tca tct tgaagaacgt acatatataa ataaatacct ttgtgcatgg tattttcagg 3553
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Ile Thr Asp Arg Phe Ile Gln Cys Leu His Asp Arg Ala Asp Pro Ser
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10 Phe Pro Ile Thr Gly Glu Val Tyr Thr Pro Gly Asn Ser Ser Phe Pro
50 55 60

Thr Val Leu Gln Asn Tyr Ile Arg Asn Leu Arg Phe Asn Glu Thr Thr
15 65 70 75 80

Thr Pro Lys Pro Phe Leu Ile Ile Thr Ala Glu His Val Ser His Ile
85 90 95

20 Gln Ala Ala Val Val Cys Gly Lys Gln Asn Arg Leu Leu Leu Lys Thr
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Arg Ser Gly Gly His Asp Tyr Glu Gly Leu Ser Tyr Leu Thr Asn Thr
115 120 125

25 Asn Gln Pro Phe Phe Ile Val Asp Met Phe Asn Leu Arg Ser Ile Asn
130 135 140

Val Asp Ile Glu Gln Glu Thr Ala Trp Val Gln Ala Gly Ala Thr Leu
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Gly Glu Val Tyr Tyr Arg Ile Ala Glu Lys Ser Asn Lys His Gly Phe
165 170 175

35 Pro Ala Gly Val Cys Pro Thr Val Gly Val Gly Gly His Phe Ser Gly
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Gly Gly Tyr Gly Asn Leu Met Arg Lys Tyr Gly Leu Ser Val Asp Asn
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40 Ile Val Asp Ala Gln Ile Ile Asp Val Asn Gly Lys Leu Leu Asp Arg
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Lys Ser Met Gly Glu Asp Leu Phe Trp Ala Ile Thr Gly Gly Gly Gly
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Val Ser Phe Gly Val Val Leu Ala Tyr Lys Ile Lys Leu Val Arg Val
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Leu Ser Thr Ile Ala Glu Arg Trp Val Gln Val Ala Asp Lys Leu Asp
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Gly Gly Lys Thr Val Arg Ala Ile Phe Pro Thr Leu Tyr Leu Gly Asn
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 Tyr Tyr Thr Gly Phe Pro Ser Gly Thr Pro Thr Thr Ala Leu Leu Ser
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 10 Arg Thr Pro Gln Arg Leu Asn Pro Phe Lys Ile Lys Ser Asp Tyr Val
 370 375 380
 Gln Asn Pro Ile Ser Lys Arg Gln Phe Glu Phe Ile Phe Glu Arg Leu
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 Lys Glu Leu Glu Asn Gln Met Leu Ala Phe Asn Pro Tyr Gly Gly Arg
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 20 Met Ser Glu Ile Ser Glu Phe Ala Lys Pro Phe Pro His Arg Ser Gly
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 Asn Ile Ala Lys Ile Gln Tyr Glu Val Asn Trp Glu Asp Leu Ser Asp
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 25 Glu Ala Glu Asn Arg Tyr Leu Asn Phe Thr Arg Leu Met Tyr Asp Tyr
 450 455 460
 Met Thr Pro Phe Val Ser Lys Asn Pro Arg Lys Ala Phe Leu Asn Tyr
 30 465 470 475 480
 Arg Asp Leu Asp Ile Gly Ile Asn Ser His Gly Arg Asn Ala Tyr Thr
 485 490 495
 35 Glu Gly Met Val Tyr Gly His Lys Tyr Phe Lys Glu Thr Asn Tyr Lys
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 55 <221> unsure
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<220>
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 <222> (379)
 <223> replace (379, "g")

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<220>
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 Pro Leu Leu Leu Val Leu Leu Ser Thr His Ser Ser Ala Thr Ser Thr
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25

tcc att ata gat cgc ttc acc caa tgt cta aac aac cga gct gac cct 144
 Ser Ile Ile Asp Arg Phe Thr Gln Cys Leu Asn Asn Arg Ala Asp Pro
 35 40 45

30

tct ttc ccg ctc agt gga caa ctt tac act ccc gat aac tcc tct ttt 192
 Ser Phe Pro Leu Ser Gly Gln Leu Tyr Thr Pro Asp Asn Ser Ser Phe
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35

cca tcc gtc ttg caa gct tac atc cgg aac ctc cga ttc aat gaa tcc 240
 Pro Ser Val Leu Gln Ala Tyr Ile Arg Asn Leu Arg Phe Asn Glu Ser
 65 70 75

40

acg act ccc aaa ccc atc tta atc atc acc gcc tta cac cct tca cac 288
 Thr Thr Pro Lys Pro Ile Leu Ile Ile Thr Ala Leu His Pro Ser His
 80 85 90

45

att caa gca gct gtt gtg tgc gcc aaa aca cac cgc ctg cta atg aaa 336
 Ile Gln Ala Ala Val Val Cys Ala Lys Thr His Arg Leu Leu Met Lys
 95 100 105 110

50

acc aga agc gga ggc cat gat tat gag ggg ctt tcc tat gtg acc aat 384
 Thr Arg Ser Gly Gly His Asp Tyr Glu Gly Leu Ser Tyr Val Thr Asn
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55

tcg aac caa ccc ttt ttt gtt gtt gac atg ttc aac tta cgc tcc ata 432
 Ser Asn Gln Pro Phe Phe Val Val Asp Met Phe Asn Leu Arg Ser Ile
 130 135 140

60

aac gtg agt att gaa gat gaa act gca tgg gtc caa gct ggt gcg act 480
 Asn Val Ser Ile Glu Asp Glu Thr Ala Trp Val Gln Ala Gly Ala Thr
 145 150 155

ctt ggt gaa gtc tac tac cga ata gca gag aaa agc aac agt cat gct 528
 Leu Gly Glu Val Tyr Tyr Arg Ile Ala Glu Lys Ser Asn Ser His Ala
 160 165 170

10

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	Gly Gly Gly Tyr Gly Asn Leu Met Gly Lys Tyr Gly Leu Ser Val Asp	
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	Asn Ile Val Asp Ala Gln Leu Ile Asp Val Asn Gly Lys Leu Leu Asn	
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	Arg Lys Ser Met Gly Glu Asp Leu Phe Trp Ala Ile Thr Gly Gly Gly	
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	Gly Val Ser Phe Gly Val Val Val Ala Tyr Lys Ile Lys Leu Val Arg	
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	Val Pro Thr Thr Val Thr Val Phe Asn Val Gln Arg Thr Ser Glu Gln	
	255 260 265 270	
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	Asn Leu Ser Thr Ile Ala His Arg Trp Ile Gln Val Ala Asp Lys Leu	
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	Asn Ser Thr Ala Leu Val Ala Leu Leu Asn Lys Asp Phe Pro Glu Leu	
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	Gly Val Glu Ile Ser Asp Cys Ile Glu Met Ser Trp Ile Glu Ser Val	
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 Val Glu Ala Ala Asn Arg Tyr Leu Asn Phe Thr Arg Val Met Tyr Asp
 450 455 460

15 tat atg act ccg ttt gtt tct aag aac ccc agg gaa gca ttt ctg aac 1440
 Tyr Met Thr Pro Phe Val Ser Lys Asn Pro Arg Glu Ala Phe Leu Asn
 465 470 475

20 tac agg gat tta gat att ggt gtc aac agt cat ggc aag aat gct tac 1488
 Tyr Arg Asp Leu Asp Ile Gly Val Asn Ser His Gly Lys Asn Ala Tyr
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 Gly Glu Gly Met Val Tyr Gly His Lys Tyr Phe Lys Glu Thr Asn Tyr
 495 500 505 510

25 aag agg cta acg atg gtg aag acg agg gtt gat cct agc aat ttt ttt 1584
 Lys Arg Leu Thr Met Val Lys Thr Arg Val Asp Pro Ser Asn Phe Phe
 515 520 525

30 agg aat gag caa agt atc cca act ttg tca tct tca tgg aag 1626
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 530 535 540

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35 ccagattcag atgatattga tataatttttg acttgatatt atacaaacaa aattatatta 1746

tattttttctg aatttagatt ttccattctt tggaaaaata tacgaacatt gatgttgata 1806

40 tttttaagaa ttatagattt tgaacattgt gaacaatgaa taaaccgagg acttcccttg 1866

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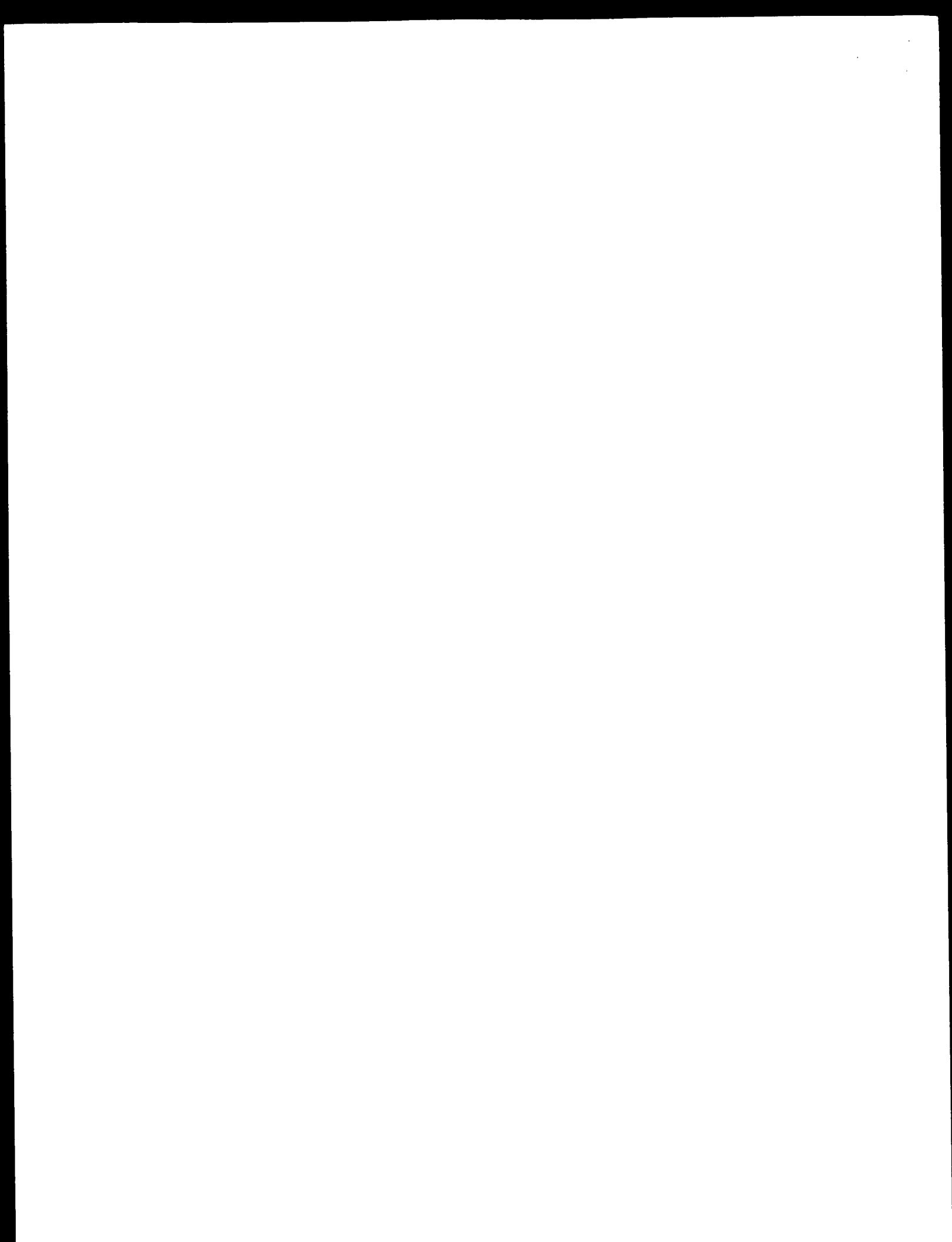
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Ile Asp Arg Phe Thr Gln Cys Leu Asn Asn Arg Ala Asp Pro Ser Phe
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Pro Leu Ser Gly Gln Leu Tyr Thr Pro Asp Asn Ser Ser Phe Pro Ser
 50 55 60
 Val Leu Gln Ala Tyr Ile Arg Asn Leu Arg Phe Asn Glu Ser Thr Thr
 5 65 70 75 80
 Pro Lys Pro Ile Leu Ile Ile Thr Ala Leu His Pro Ser His Ile Gln
 85 90 95
 10 Ala Ala Val Val Cys Ala Lys Thr His Arg Leu Leu Met Lys Thr Arg
 100 105 110
 Ser Gly Gly His Asp Tyr Glu Gly Leu Ser Tyr Val Thr Asn Ser Asn
 115 120 125
 15 Gln Pro Phe Phe Val Val Asp Met Phe Asn Leu Arg Ser Ile Asn Val
 130 135 140
 Ser Ile Glu Asp Glu Thr Ala Trp Val Gln Ala Gly Ala Thr Leu Gly
 20 145 150 155 160
 Glu Val Tyr Tyr Arg Ile Ala Glu Lys Ser Asn Ser His Ala Phe Pro
 165 170 175
 25 Ala Gly Val Cys Pro Thr Val Gly Val Gly Gly His Phe Ser Gly Gly
 180 185 190
 Gly Tyr Gly Asn Leu Met Gly Lys Tyr Gly Leu Ser Val Asp Asn Ile
 195 200 205
 30 Val Asp Ala Gln Leu Ile Asp Val Asn Gly Lys Leu Leu Asn Arg Lys
 210 215 220
 Ser Met Gly Glu Asp Leu Phe Trp Ala Ile Thr Gly Gly Gly Gly Val
 35 225 230 235 240
 Ser Phe Gly Val Val Val Ala Tyr Lys Ile Lys Leu Val Arg Val Pro
 245 250 255
 40 Thr Thr Val Thr Val Phe Asn Val Gln Arg Thr Ser Glu Gln Asn Leu
 260 265 270
 Ser Thr Ile Ala His Arg Trp Ile Gln Val Ala Asp Lys Leu Asp Asn
 275 280 285
 45 Asp Leu Phe Leu Arg Met Thr Phe Asn Val Ile Asn Asn Thr Asn Gly
 290 295 300
 Glu Lys Thr Ile Arg Gly Leu Phe Pro Thr Leu Tyr Leu Gly Asn Ser
 50 305 310 315 320
 Thr Ala Leu Val Ala Leu Leu Asn Lys Asp Phe Pro Glu Leu Gly Val
 325 330 335
 55 Glu Ile Ser Asp Cys Ile Glu Met Ser Trp Ile Glu Ser Val Leu Phe
 340 345 350
 Tyr Thr Asn Phe Pro Ile Gly Thr Pro Thr Thr Ala Leu Leu Ser Arg
 355 360 365

Thr Pro Gln Arg Leu Asn Pro Phe Lys Ile Lys Ser Asp Tyr Val Lys
 370 375 380
 Asn Thr Ile Ser Lys Gln Gly Phe Glu Ser Ile Phe Glu Arg Met Lys
 5 385 390 395 400
 Glu Leu Glu Asn Gln Met Leu Ala Phe Asn Pro Tyr Gly Gly Arg Met
 405 410 415
 10 Ser Glu Ile Ser Glu Phe Ala Lys Pro Phe Pro His Arg Ser Gly Asn
 420 425 430
 Ile Ala Lys Ile Gln Tyr Glu Val Asn Trp Asp Glu Leu Gly Val Glu
 435 440 445
 15 Ala Ala Asn Arg Tyr Leu Asn Phe Thr Arg Val Met Tyr Asp Tyr Met
 450 455 460
 Thr Pro Phe Val Ser Lys Asn Pro Arg Glu Ala Phe Leu Asn Tyr Arg
 20 465 470 475 480
 Asp Leu Asp Ile Gly Val Asn Ser His Gly Lys Asn Ala Tyr Gly Glu
 485 490 495
 25 Gly Met Val Tyr Gly His Lys Tyr Phe Lys Glu Thr Asn Tyr Lys Arg
 500 505 510
 Leu Thr Met Val Lys Thr Arg Val Asp Pro Ser Asn Phe Phe Arg Asn
 515 520 525
 30 Glu Gln Ser Ile Pro Thr Leu Ser Ser Ser Trp Lys
 530 535 540
 35

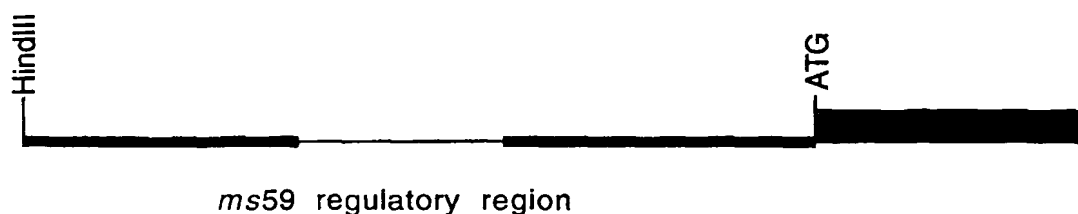




INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(74) Agent: VAN WEZENBEEK, Lambertus, A., C., M.; Mogen International N.V., P.O. Box 628, NL-2300 AP Leiden (NL).		(88) Date of publication of the international search report: 23 December 1999 (23.12.99)	

(54) Title: PATHOGEN-INDUCIBLE PROMOTER



— = part sequenced

- - - = ms59 coding region

(57) Abstract

This invention describes pathogen-inducible promoters which normally drive expression of plant hexose oxidases, especially those which can be isolated from *Helianthus annuus* and *Lactuca sativa*, more specifically those promoters which naturally are the regulatory regions driving expression of the hexose oxidase MS59 and WL64, respectively. Also claimed are chimeric constructs where these pathogen-inducible promoters drive expression of antipathogenic proteins or of proteins which can elicit a hypersensitive response.

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INTERNATIONAL SEARCH REPORT

International Application No

PC1/EP 99/02178

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/82 C12N5/10 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FACCHINI, P.J., ET AL.: "molecular characterization of berberine bridge enzyme genes from Opium poppy" PLANT PHYSIOLOGY, vol. 112, 1996, pages 1669-1677, XP002111516 the whole document	1,7,8, 12-15,20
Y	HAUSCHILD, K., ET AL.: "isolation and analysis of a gene bbel encoding the berberine bridge enzyme from California poppy Eschscholzia californica" PLANT MOLECULAR BIOLOGY, vol. 36, February 1998 (1998-02), pages 473-478, XP002111517 the whole document	1,7,8, 12-15,20

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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- "&" document member of the same patent family

Date of the actual completion of the international search

15 October 1999

Date of mailing of the international search report

28.10.99

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Holtorf, S

INTERNATIONAL SEARCH REPORT

International Application No

PC1/EP 99/02178

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DITTRICH, H. AND KUTSCHAN, T.M.: "MOLECULAR CLONING, EXPRESSION, AND INDUCTION OF BERBERINE BRIDGE ENZYME, AN ENZYME ESSENTIAL TO THE FORMATION OF BENZOPHENANTHRIDINEALKALOIDS IN THE RESPONSE OF PLANTS TO PATHOGENIC ATTACK" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA, vol. 88, 1991, pages 9969-9973, XP002025475 the whole document	1,7,8, 12-15,20
A	WO 96 34949 A (COMMW SCIENT IND RES ORG ;UNIV AUSTRALIAN (AU); PRYOR ANTHONY J (A) 7 November 1996 (1996-11-07) abstract, page 6,7,9,20; examples	1-22
A	WO 96 28561 A (MAX PLANCK GESELLSCHAFT ;STRITTMATTER GUENTER (DE)) 19 September 1996 (1996-09-19) pages 1-2,5-7,11; page 15, last paragraph; pages 16,17; examples	1-22
A	EP 0 337 532 A (MOGEN INT ;UNIV LEIDEN (NL)) 18 October 1989 (1989-10-18) column 3, 4, line 26-32; column 6, line 23-42; claims	1-22
A	EP 0 307 841 A (GEN HOSPITAL CORP) 22 March 1989 (1989-03-22) pages 3,5,6,16; claims	1-22
A	WO 98 03536 A (LEBEL EDOUARD GUILLAUME ;RYALS JOHN ANDREW (US); WARD ERIC RUSSELL) 29 January 1998 (1998-01-29) pages 1-5; page 9, line 8-17; claims	1-22
P,X, L	WO 98 13478 A (SELA BUURLAGE MARIANNE BEATRIX ;MELCHERS LEO SJOERD (NL); STUIVER) 2 April 1998 (1998-04-02) page 6, line 37 - page 7, line 5; page 21, line 32 - page 22, line 15; examples;1,4; claims 47-50. ALSO: BBE enzyme is shown to exhibit Hexose Oxidase activity	1-18,20, 22

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 99/02178

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
A meaningful search for the aspect concerning the promoter region of the clone WL64 was not possible due to insufficient disclosure of the subject matter, i.e. no sequence data were provided.. The search was limited to a keyword search.
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

As a result of the prior review under R. 40.2(e) PCT,
all additional fees are to be refunded.

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☒ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/EP 99/02178

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

A meaningful search for the aspect concerning the promoter region of the clone WL64 was not possible due to insufficient disclosure of the subject matter, i.e. no sequence data were provided.. The search was limited to a keyword search.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PC1/EP 99/02178

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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